American Chemistry Council

July 19, 2019

EPA-HQ-OPPT-2019-0238
OPPT Docket
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

Re: Draft Risk Evaluation for 1,4-Dioxane - CASRN:123-91-1, EPA-740-R1-8807 (June 2019); for consideration by the Science Advisory Committee on Chemicals

To Whom It May Concern:

The 1,4-Dioxane Panel (Panel) of the American Chemistry Council appreciates the opportunity to provide the enclosed comments to the Science Advisory Committee on Chemicals (SACC) on the draft risk evaluation for 1,4-dioxane. The Panel includes companies with an interest in the interpretation of the scientific information available for 1,4-dioxane. As such, we are very concerned that the draft risk evaluation fails to fully consider the clear evidence for key events supporting a threshold of carcinogenic response in animals exposed to the chemical.

1,4-Dioxane is among the first ten chemicals to be identified as a priority substance under the 2016 amendments to the Toxic Substances Control Act (TSCA). The Office of Pollution Prevention and Toxics' (OPPT) review of the substance, consequently, has been complicated by the need to develop standard practices for conducting a weight of evidence evaluation based on a systematic review of the best available science and other data as required by the statute and implementing regulations.[1] The 1,4-dioxane draft now before the SACC is one of the first two draft evaluations for a data-rich chemical to result from the Agency's evolving risk evaluation process and the first to suggest that certain conditions of use may present an unreasonable risk to health.

Because of the significance of the draft risk evaluation to the Agency's ongoing risk assessment and risk management approach, and the importance of this Committee's review, the Panel is disappointed in OPPT's decision to curtail stakeholder input prior to the SACC's consideration. Given the limited time that we have been provided to review the draft and

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[1] 82 *Fed Reg* 33726. Procedures for chemical risk evaluation under the amended Toxic Substances Control Act. Final Rule (July 20, 2017).

prepare comments for discussion with the SACC, our comments are focused on the human health hazard assessment described in the draft risk evaluation. We have not conducted detailed reviews of the systematic review or exposure assessment information that are critical parts of the evaluation. Nor have we reviewed the draft risk characterizations for the identified conditions of use. We plan to conduct a more thorough review in the coming weeks.

In light of the significance of the draft risk evaluation for 1,4-dioxane to EPA's ongoing, and still evolving, approach to chemical management, the Panel urges EPA to allow the SACC to conduct a robust and informed peer review of the draft. The health effects information available for 1,4-dioxane raises fundamental scientific questions that require careful deliberation and that can be informed by stakeholder input. We hope that you will take the necessary time to receive input from ACC and others as you evaluate these questions.

Sincerely,

Steve Risotto

Stephen P. Risotto
Senior Director

Enclosure

1,4-Dioxane Panel of the American Chemistry Council
Comment to the Science Advisory Committee on Chemicals
on the Draft Risk Evaluation for 1,4-Dioxane

July 19, 2019

Executive Summary

In the draft risk evaluation for 1,4-dioxane, EPA concludes that the evidence for a threshold mode of action is not sufficiently robust and defaults to the genotoxic mode of action (MOA) in characterizing risk from 1,4-dioxane exposure. Unfortunately the EPA fails to fully consider the considerable evidence for the key events supporting a threshold for carcinogenic response in animals exposed to 1,4-dioxane.

Based on the currently available evidence, the genotoxic MOA is inappropriate primarily because 1,4-dioxane is not genotoxic. This conclusion is based on extensive testing with *in vitro* assay systems with prokaryotic organisms, non-mammalian eukaryotic organisms, mammalian cells, and *in vivo* genotoxicity assays. In addition, there is ample evidence that the development of tumors only occurs when dosing exceeds the threshold of metabolic saturation (Figure 1). Metabolism studies confirm that, while the substance is readily metabolized and quickly eliminated from the body, the metabolic pathway becomes saturated at higher exposure levels of 1,4-dioxane. Moreover, available evidence demonstrates that toxicity occurs only after the clearance pathway becomes saturated and the parent compound accumulates in the blood. Thus, there is ample evidence to support a threshold MOA when assessing risks from exposure to 1,4-dioxane.

Although 1,4-dioxane has been reported to evoke multiple tumors, the increased tumor incidences tend to occur at the highest dose only, and all are consistent with a threshold-based non-mutagenic mode of action. Chronic and subchronic studies in laboratory animals exposed to levels above metabolic saturation have consistently demonstrated a threshold response to tumor formation from 1,4-dioxane exposure. This has been recognized by authoritative bodies worldwide and has led authorities in Australia, Canada, Europe, and Japan to apply threshold assumptions when characterizing risk. Despite this abundance of information and precedence for the threshold approach, the draft risk evaluation inexplicitly employs a default linear low-dose extrapolation to develop a unit risk estimate and cancer slope factor based on benchmark dose modeling for multiple tumor sites.

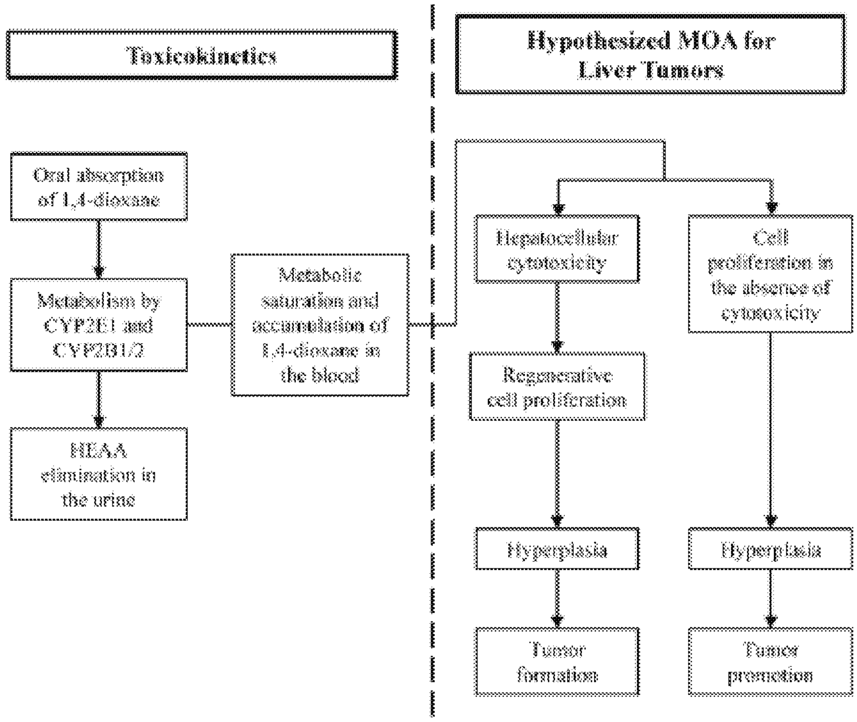

Figure 1. Identification of key events in liver tumor formation following exposure to 1,4-dioxane[1]

Genotoxicity Data

1,4-Dioxane has been tested for genotoxicity using *in vitro* and *in vivo* testing with prokaryotic organisms, non-mammalian eukaryotic organisms, and mammalian cells, both with and without metabolic activation. Based on the data presented in USEPA's 2013 Integrated Risk Information System (IRIS) assessment, all fifteen mutagenicity tests reported were negative.[2] In addition, 22 *in vitro* genotoxicity assays and nine *in vivo* genotoxicity assays were negative. Although eight assays were noted to be positive, genotoxicity was only observed at high and noted cytotoxic doses. Such genotoxicity outcomes at cytotoxic doses, such as increased micronuclei, are the result of cell injury and not mutation. Based on these result, the IRIS assessment concluded that 1,4-dioxane is not genotoxic in the absence of cytotoxicity. The 2019 draft risk evaluation supports the conclusion that 1,4-dioxane is not genotoxic and does not induce tumors via a mutagenic mode of action. However, the draft suggests that recent studies concerning the genetic toxicity of the substance at elevated exposures and data from in vitro

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1 USEPA IRIS 2013, at 95.

2 USEPA. Toxicological review of 1,4-Dioxane (with inhalation update) (CAS No. 123-91-1) in support of summary information on the Integrated Risk Information System (IRIS.) EPA-635/R-11/003-F. Washington, DC (2013), at 73.

screening assays (ToxCast) may be indicative of DNA damage. As described below, this is an incorrect interpretation of EPA's own data.

While 1,4-dioxane appears to stimulate DNA synthesis in rats at elevated exposure levels, $\geq$ 1000 milligrams per kilogram (mg/kg) per day, it does not represent a genotoxic event. Rather, the DNA synthesis appears to be a key event for a regenerative cell proliferation and/or tumor promotion and can occur in either the presence or absence of cytotoxicity.[3] The DNA synthesis evidence supports the conclusion that 1,4-dioxane promotes cell proliferation, through mitogenesis and/or cytotoxicity depending on the dose and target organ.

<u>Recent Genetic Toxicity Studies</u>

Recent reports of a marginal increase in mutation frequency at high doses by Itoh and Hattori[4] and Gig *et al.*, [5] while of interest, should not be viewed as providing evidence for a genotoxic cancer mode of action (MoA), and certainly not one associated with low-dose exposures. This is particularly evident as the doses used in both studies exceed the saturation kinetics of the pathway for metabolism of 1,4-dioxane in the rat -- estimated to occur between 30 and 100 milligrams per kilogram body weight (mg/kg) per day.[6] In fact, Gi *et al.* stress the presence of no-effect levels for both mutagenicity and carcinogenicity of 1,4-dioxane.

Increased mutation is a common result of exposures to non-genotoxic carcinogens that exceed the threshold for effects. [7] Earlier work with the same transgenic rat strain used by Gi *et al.* noted that the length of exposure required for preneoplastic hepatic foci formation "may increase the risk of false-positive results of mutations due to nongenotoxic mechanisms caused by chronic toxicity."[8] This effect is exacerbated by the high doses of 1,4-dioxane required to generate genotoxicity which have been shown to interfere with cell proliferation in the liver.[9] As a result, Morita and Hayashi (1998) concluded that "[i]t is also conceivable that the positive

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[3] Goldsworthy TL *et al.* Examination of potential mechanisms of carcinogenicity of 1,4-dioxane in rat nasal epithelial cells and hepatocytes. *Arch Toxicol* 65: 1-9 (1991).

[4] Itoh S Hattori C. *In vivo* genotoxicity of 1,4-dioxane evaluated by liver and bone marrow micronucleus tests and *Pig-a* assay in rats. *Mutat Res Gen Toxicol* 837:8-14 (2019).

[5] Gi M *et al. In vivo* positive mutagenicity of 1,4-dioxane and quantitative analysis of its mutagenicity and carcinogenicity in rats. *Arch Toxicol* 92:3207-3221 (2018).

[6] The doses in the study by Gi *et al.* (2018) were 250 and 1250 mg/kg; Itoh and Hattori (2019) dosed the animals at 1000 to 3000 mg/kg.

[7] Singh VK *et al.* Comparison of the mutant frequencies and mutation spectra of three non-genotoxic carcinogens, oxazepam, phenobarbital, and Wyeth 14,643, at the λcII locus in Big Blue® transgenic mice. *Biochem Pharmacol* 62(6):685-92 (2001).

[8] Thybaud V *et al.* In vivo transgenic mutation assays. *Mutat Res* 540:141–151 (2003).

[9] Roy *et al.* Chromosome breakage is primarily responsible for the micronuclei induced by 1,4-dioxane in the bone marrow and liver of young CD-1 mice. *Mut Res Gen Toxicol Environ Mut* 586(1):28-37 (2005).

result in mouse liver micronucleus assay was due to a nongenotoxic mechanism, i.e., errors in genetic repair following enhancement of hepatocyte proliferation."[10] This same mechanism – increased regenerative proliferation leading to a higher background level of clastogenic effects, has also been shown to be at play for compounds triggering haematotoxin effects, signifying it as indirect, non-DNA reactive genotoxicity that have a threshold, as is the case for 1,4-dioxane.[11]

<u>ToxCast Results</u>

The draft risk evaluation states that "1,4-dioxane was observed to increase the transcriptional activity of the p53 tumor suppressor protein in human colon cancer cells (HCT116) 24 hours after 1,4-dioxane exposure" and suggests that this may be "indicative of an active DNA damage and repair response." As described further below, the results in ToxCast are completely negative in all 113 assays that were run for 1,4-dioxane, and do *not* support a conclusion that there is any activity associated with DNA damage and repair.

In the ToxCast analysis, p53 transcriptional activity is evaluated in five sequential assays,[12] done over six sequential months to evaluate the stability of the test plates over time.[13] While the CompTox dashboard indicates a "hit" in the fourth of the five sequential assays, it is accompanied with a data quality flag. The data is flagged since only one data point suggests a response while all the other points indicate no response. There is no basis to interpret this single point finding in the middle of a sequential series of repeated assays as evidence of relevant biological activity.

Metabolism & Toxicokinetics

Metabolism of 1,4-dioxane in humans and experimental animals is well characterized and extensive. In both rodents and humans, 1,4-dioxane is metabolized by cytochrome P-450 (P450) to β-hydroxyethoxy acetic acid (HEAA) in a linear, first-order process.[14] This metabolic transformation is responsible for the rapid clearance of 1,4-dioxane and elimination in the urine. Induction of P450 isoforms has been observed in the liver, kidney, and nasal mucosa in

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[10] Morita T and Hayashi M. 1,4-dioxane is not mutagenic in five *in vitro* assays and mouse blood micronucleus assay, but is in mouse liver micronucleus assay. *Environ Molec Mut* 32(3):269-280 (1998).

[11] Tweats DJ *et al.* Report of the IWGT working group on strategy/interpretation for regulatory *in vivo* tests: II. Identification of *in-vivo* only positive compounds in the bone marrow micronucleus test. Mutat Res 627(1):78-91 (2007).

[12] The assays are identified as p53_BLA_p1_ratio, p2, p3, p4 and p5.

[13] Personal communication from Richard Judson, USEPA, April 29, 2019.

[14] Young JD *et al.* The dose-dependent fate of 1,4-dioxane in rats. *J. Environ Pathol Toxicol* 2:263-282 (1978).

rats exposed either by gavage (acute) or in drinking water (chronic).[15] Workers exposed to 1,4-dioxane at concentrations up to 50 parts per million (ppm) showed a linear elimination of 1,4-dioxane in both plasma and urine leading to the metabolite HEAA.[16] Studies in rats dosed by gavage up to 1000 mg/kg did not detect another suggested metabolite, 1,4-dioxane-2-one, leading the researchers to conclude that it was not formed or that it quickly hydrolyzed to HEAA.[17]

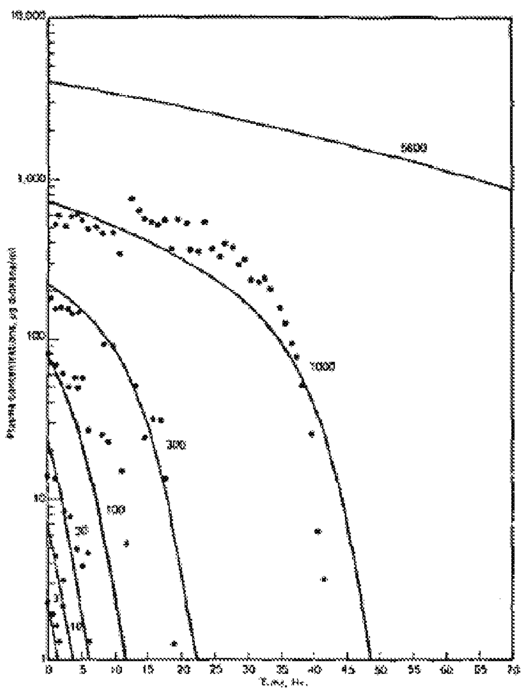

Figure 2. Plasma concentrations over time for various intravenous doses from 3 to 1000 mg/kg of 1,4-dioxane administered to rats[18]

At higher doses of 1,4-dioxane in experimental animals the metabolism of 1,4-dioxane shifts from linear, first-order metabolism to a zero-order kinetics indicating metabolic saturation. This kinetic pattern has been demonstrated directly by monitoring plasma levels after intravenous (IV) administration of 1,4-dioxane and indirectly from studies monitoring the elimination of HEAA in the urine.[15] Rats given IV 1,4-dioxane demonstrated a dose-related shift

15 Nannelli A *et al.* Effects of dioxane on cytochrome P450 enzymes in liver, kidney, lung and nasal mucosa of rat. *Arch Toxicol* 79, 74-82 (2005).

16 Young JD *et al.* (1977). Pharmacokinetics of 1,4-dioxane in humans. *J Toxicol Environ Health* 3:507-520 (1977).

17 US Army Public Health Command. Studies on metabolism of 1,4-dioxane. Toxicology Report No. 87-XE-08WR-09. Aberdeen Proving Ground, MD (March 2010).

18 Young JD *et al.* Dose-dependent fate of 1,4-dioxane in rats. *J Toxicol Environ Health* 4:709 -726 (1978).

from linear to saturation metabolism of 1,4-dioxane at 30 to 100 mg/kg/day resulting in an increase in 1,4-dioxane blood levels (Figure 2). Similarly, rats given gavage doses of 10, 100, or 1000 mg/kg showed that the plasma clearance rate decreased with dose, while the fraction excreted as HEAA decreased and the fraction excreted as the parent compound increased. For mice, saturation of the P450 pathway appears to start at 200 mg/kg.[19] Saturation of the metabolic pathway also has been observed in human hepatocytes exposed to up to 25 mg/ml of 1,4-dioxane (Figure 3).

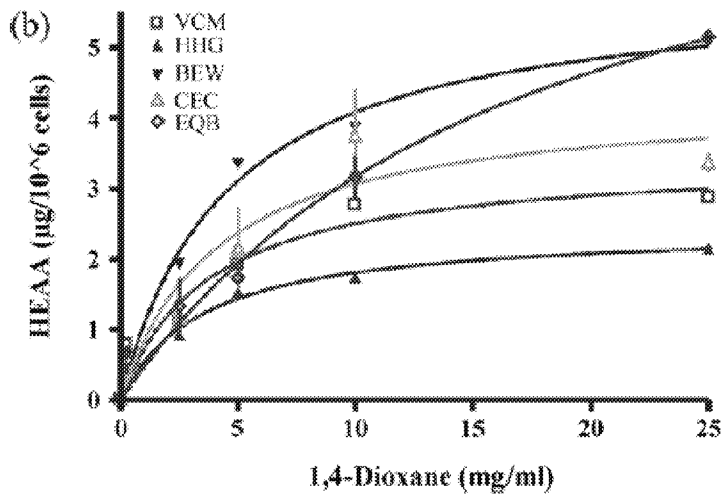

Figure 3. Production of HEAA in hepatocytes from human donors incubated with initial concentrations of 1,4-dioxane from 0.25 to 25 mg/ml[20]

Once saturated, increased exposures result in a disproportional increase in circulating levels of 1,4-dioxane. This saturation phenomenon was observed in female mice exposed to up to 6000 ppm (1000 mg/kg/day) in drinking water for up to 90 days in a recently completed subchronic study sponsored by ACC (Figure 4).[21] At days 7 and 28, 1,4-dioxane blood concentrations were near or below the limit of detection. By day 90, blood levels of the parent compound increased significantly – but only at the highest dosage. At the same time, HEAA blood concentrations declined from day 7 to day 28, and by day 90, remained suppressed indicating a shift downward in the oxidative metabolism of 1,4-dioxane to HEAA.

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[19] Sweeney LM *et al.* Physiologically based pharmacokinetic modeling of 1,4-dioxane in rats, mice, and humans. *Tox Sci* 101(1):32-50 (2008). http://dx.doi.org/10.1093/toxsci/kfm251

[20] Sweeney *et al.* 2008, at 38.

[21] Toxicology and Environmental Research and Consulting (TERC). Investigating the mode of action for 1,4-dioxane-induced liver tumors in B6D2F1/Crl mice. Midland, MI. (2019). Report to be submitted to EPA.)

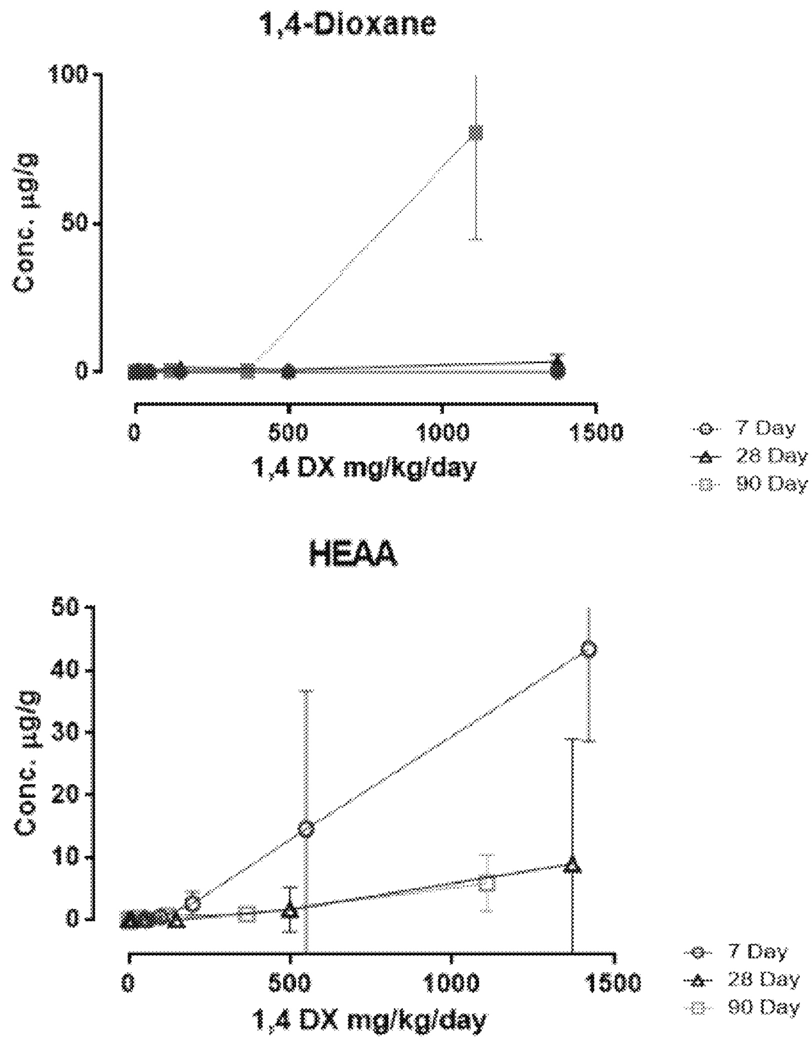

Figure 4. Blood levels of 1,4-dioxane (1,4 DX) and HEAA in female Crl/DDF1 mice exposed to up to 6000 ppm of 1,4-dioxane in drinking water (ACC)

The results from the ACC sponsored study are summarized below and will be submitted to EPA for review. They are consistent with changing metabolic competency of the female mice as a critical key event in 1,4-dioxane toxicity.[22]

[22] According to EPA's Guidelines for Carcinogen Risk Assessment, a key event is defined as "an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element." EPA/630/P-03/001F (March 2005) at 1-10.

Toxicokinetics

Studies have been conducted to determine whether 1,4-dioxane or one or more of its metabolites contributes to the chemical's reported toxicity. A study investigating the role of CYP450 isozymes in the liver toxicity of 1,4-dioxane reported no change in plasma alanine aminotransferase (ALT) activity or hepatic glutathione content even after inducing CYP with phenobarbital or by fasting – indicating that HEAA or any potentially highly reactive intermediates do not play a role in the liver toxicity of 1,4-dioxane.[23] Pretreatment with inducers of mixed-function oxidases also did not significantly change the extent of covalent binding in subcellular fractions, supporting the conclusion that the metabolites are not toxicologically active.[24]

Taken together, these data support the hypothesis that the parent compound, 1,4-dioxane, and not a metabolite, is the toxic moiety. The observations in rat studies, and most of the studies in mice, support the conclusion that liver toxicity only occurs at exposures that exceed the metabolic threshold.[25,26]. It is therefore reasonable to conclude that metabolic saturation is an essential early key event to enable the subsequent sequence of events leading to tumor formation.[27,28]

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23 Nanelli A *et al.* Effects of dioxane on cytochrome P450 enzymes in liver, kidney, lung and nasal mucosa of rat. *Arch Toxicol* 79:74-82 (2005).

24 Woo Y *et al.* Metabolism in vivo of dioxane: effect of inducers and inhibitors of hepatic mixed-function oxidases. *Biochem Pharmacol* 26:1539-1542 (1977).

25 Dourson M *et al.* Mode of action analysis for liver tumors from oral 1,4-dioxane exposures and evidence-based dose response assessment. *Regul Toxicol Pharma* 68:387-401 (2014).

26 Dourson M *et al.* Update: Mode of action (MOA) for liver tumors induced by oral exposure to 1,4-dioxane. *Regul Toxicol Pharma* 88:45-55 (2017).

27 Julien E *et al.* The key events dose-response framework: a cross-disciplinary mode-of-action based approach to examining dose-response and thresholds. *Critical Rev Food Sci Nutr 49*(8), 682–689 (2009).

28 Boobis AR *et al.* Application of key events analysis to chemical carcinogens and noncarcinogens. *Crit. Rev. Food Sci Nutr* 49:690–707 (2009).

This is the position taken by Heath Canada,[29] the World Health Organization,[30] the Australian National Industrial Chemicals Notification and Assessment Scheme,[31] and the Health Council of the Netherlands[32] in their assessments of health risks from 1,4-dioxane exposure.

Discussion of Cancer Bioassay Results

The draft risk evaluation reviews data available from three studies in drinking water in laboratory animals and one inhalation study in male rats for its analysis of the potential carcinogenicity of 1,4-dioxane. Of the drinking water studies, the study by Kano *et al.* in DuCrj rats was selected for use in the draft's risk characterization over the study by Kociba *et al.*[33] The third study by the National Cancer Institute (NCI) was considered to be low quality by the Agency and was not used. The study by Kasai *et al.* was used as the basis for assessing inhalation cancer risks; a second study by Torkelson *et al.*[34] which exposed animals to a single but was not considered in the draft evaluation. For characterizing potential risks from dermal exposure, the draft evaluation conducted route-to-route extrapolations from the Kano *et al.* and Kasai *et al.* studies. Even though EPA selected specific findings from the drinking water and inhalation studies of Kano et al., 2009 and Kasai et al., 2009; important key event and MOA information is available in the other cancer bioassays. Furthermore, a number of mechanistic studies looking at increased DNA synthesis and tumor promotion, add additional evidence supporting key events involving increased cell proliferation and tissue injury.

The 2005 Guidelines for Carcinogen Risk Assessment emphasize that, "[r]ather than viewing default options as the starting point from which departures may be justified by new scientific information, these cancer guidelines view a critical analysis of all of the available information that is relevant to assessing the carcinogenic risk as the starting point from which a default option may be invoked if needed to address uncertainty or the absence of critical information."[35] Yet, this is *not* what has been done in EPA's 2019 draft risk evaluation for 1,4-

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[29] Health Canada. 1,4-Dioxane in drinking water. Guideline Technical Document for Public Consultation (2018).

[30] WHO. 1,4-Dioxane in Drinking Water. Background document for development of WHO Guideline for Drinking Water Quality. WHO/SDE/WSH/05.08/120 (2005).

[31] NICNAS. Priority Existing Chemical Assessment Reports: 1,4-dioxane. Australia Department of Health and Ageing, Sydney, Australia. June (1998).

[32] Health Council of the Netherlands. 1,4-Dioxane – re-evaluation of the carcinogenicity and genotoxicity (2015). https://www.healthcouncil.nl/documents/advisory-reports/2015/11/13/14-dioxane-re-evaluation-of-the-carcinogenicity-and-genotoxicity

[33] Although the data quality of both the Kano *et al.* and Kociba *et al.* studies was considered high, the draft evaluation suggests that mortality of the animals at high doses in the Kociba *et al.* study limited its usefulness (see page 149).

[34] Torkelson TR *et al.* 1,4-Dioxane. II. Results of a 2-year inhalation study in rats. *Toxicol Appl Pharmacol* 30: 287-298 (1974). http://dx.doi.org/10.1016/0041-008X(74)90100-8

[35] Guidelines for Carcinogen Risk Assessment, at 1-7.

dioxane. The weight of evidence clearly supports that the mode of action for rodent tumors associated with high doses of 1,4-dioxane does not include the potential for mutagenicity, and the science clearly supports a threshold for both noncancer and cancer effects.

In the description of the liver tumors, the draft risk evaluation acknowledges that "there is little evidence for mutagenicity, but there is some evidence that 1,4-dioxane may be genotoxic at high doses."[36] In fact, as described above, there is substantial evidence that 1,4-dioxane is *not* genotoxic at the low and mid doses in the rodent bioassays, and the possibility that there could be a genotoxic component to the mode of action associated with extremely high doses (e.g., 5000 ppm) is not relevant to the lower doses. Rather than a critical evaluation of the science and underlying biology, EPA has simply modeled everything and then chosen the most conservative number. The Agency acknowledges that "MS-Combo was applied twice to evaluate uncertainties related to model choice and mechanisms: one MS-Combo model run included all tumors, while an additional model run excluded liver tumors." It is not clear why this was done since the Agency simply chose the most conservative value associated with linear low-dose extrapolation in the end.

<u>Rat Oral Bioassay (Kano *et al.* 2009)</u>

In the draft risk evaluation, EPA modeled four tumor types found at an increased incidence in male rats in the Kano *et al.* drinking water bioassay: nasal squamous cell carcinoma, peritoneal mesothelioma, hepatocellular adenoma or carcinoma, and subcutis fibroma. The only statistically significant increase in tumors was seen at the highest dose of 5000 ppm for peritoneal mesothelioma and hepatocellular adenoma/carcinoma. No statistically significant increase was reported for nasal carcinomas or subcutis fibroma. A critical evaluation of each of these tumor types follows.

Table 1. Tumor data reported by Kano *et al.*

Tumor Type	Controls (50/group)	200 ppm (50/group)	1000 ppm (50/group)	5000 ppm (50/group)
nasal squamous cell carcinoma	0	0	0	3
peritoneal mesothelioma	2	2	5	28*
hepatocellular adenoma or carcinoma	3	4	7	39*
subcutis fibroma	5	3	5	12

* statistically significant, $p<0.01$

36 Draft Risk Evaluation for 1,4-Dioxane, at 98

Nasal Squamous Cell Carcinomas (SCC)

EPA extrapolated the observable tumor incidence data and inappropriately applied a linear extrapolation (Figure 5). In the Kano *et al.* bioassay, there were 0/50 nasal SCC in control male rats, 0/50 in the low-dose and 0/50 in the mid-dose group. The high dose group had only 3/50 tumors (6%). Similar results were seen in the female rats by Kano *et al.* and in the drinking water bioassay by Kociba *et al.* (1974). In that study, Kociba *et al.* reported no nasal tumors seen in control male rats or in rats administered 100 ppm in drinking water (9.6 mg/kg/day) or 1000 ppm (94 mg/kg/day); at the highest dose of 10,000 ppm (1015 mg/kg/day), 3/66 male rats developed nasal tumors.

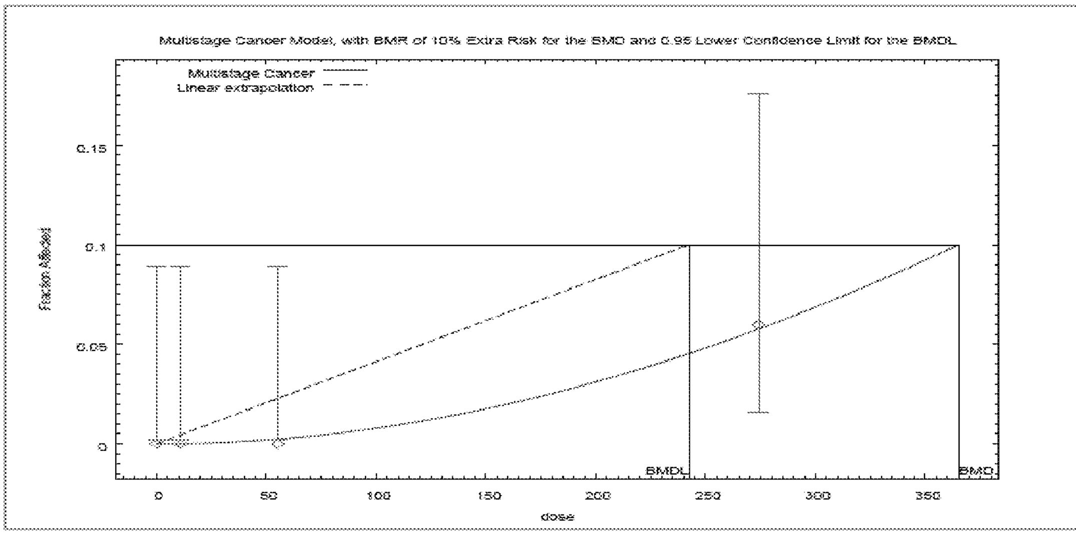

Figure 5. Fitted curve for Multistage-Cancer 2 model for
Nasal squamous cell carcinoma in Male F344/DuCrj rats from Kano *et al.* 2009
(Figure I-14 of draft risk evaluation, p 376)

These tumors are clearly associated with a threshold, whether through internal exposure or by direct contact from drinking water. [37]

Peritoneal Mesothelioma

Peritoneal mesothelioma are well-recognized as a common, age-related spontaneous tumor in the male F344 rat, clearly acknowledged by the study authors: "It has been recognized that the peritoneal mesothelioma is a commonly observed, spontaneous neoplasm of male F344 rats arising from the tunica vaginalis." The only statistically-significant increase seen in the Kano *et al.* bioassay was at the highest dose of 5000 ppm. It is also noted that this tumor type was *not*

[37] Agency for Toxic Substances and Disease Registry. Toxicological profile for 1,4-dioxane. U.S. Department of Health and Human Services, Public Health Service. Atlanta, GA (2012).

increased in the drinking water bioassay in male rats by Kociba *et al.* (1974). This provides clear evidence for a threshold for this tumor type and linear low-dose extrapolation is not supported by the science.

Subcutis Fibromas

These fibromas also are well-recognized as one of the most common, age-related spontaneous and nonmalignant tumors in the male F344 rat, including in Japanese bioassays (Maita et al., 1987). As with the nasal tumors, there was no increase in the low- or mid-dose male rats, and a non-statistically significant increase at the high dose. It is also noteworthy that in female rats data reported by Kano *et al.*, the incidence of these tumors was 0/50, 2/50, 1/50, 0/50 for controls, low, mid, and high dose groups. While it is common to see sex-related differences in tumor incidences, the inverted trend in the female rats helps substantiate the clear threshold for this tumor type, if it is related to 1,4-dioxane at all.

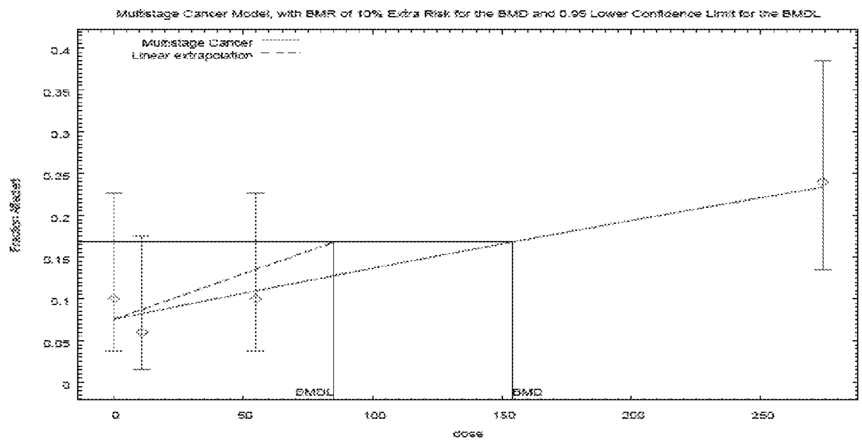

Figure 6. Fitted curve for Multistage-Cancer 1° model for
Subcutis fibroma in Male F344/DuCrj rats from Kano *et al.* 2009
(Figure I-17 of draft risk evaluation, p 382)

This is also recognized by Kano *et al.* who note that "the subcutis fibroma and mammary gland adenoma and fibroadenoma are benign, commonly observed and spontaneous neoplasms of F344 rats." It is further noted that in the abstract of the published bioassay, Kano et al. (2009) did not point to an association between subcutis fibromas in the male rats and 1,4-dioxane exposure. Thus, modeling of the subcutis fibromas and application of linear low-dose extrapolation is entirely inappropriate. This is confirmed by visual inspection of the dose-response curve in Figure 6.

Hepatocellular Adenoma or Carcinoma

As with the other tumor types, an increase in hepatocellular tumors was only seen at the highest dose of 5000 ppm. In fact, there were 0/50 carcinomas in controls, low, and mid-dose rats. The increase at the high-dose is clearly associated with 1,4-dioxane treatment, mediated by a non-genotoxic MOA with a clear threshold. This is also supported by the drinking water bioassay by Kociba *et al.* in which an increase in liver tumors was seen only at a very high dose (10,000 ppm):

Table 2. Hepatic tumors reported by Kociba *et al.*

Tumor Type	Controls (106/group)	100 ppm (110/group)	1000 ppm (106/group)	10,000 ppm (66/group)
Hepatic tumors, All types	2	0	1	12
Hepatocellular carcinomas	1	0	1	10

Kano *et al.* also reported on the incidence of altered hepatocellular foci, considered to be a preneoplastic lesion. While there was a statistically significant increase in mixed cell foci at the mid-dose (1000 ppm), there was a *decreased* incidence of acidophilic foci (7/50) in the same dose group compared to controls (12/50), such that overall there was no overall increase in altered hepatocellular foci until the high dose. Kano *et al.* also report a significant increase in liver weight in mid- and high-dose male rats, with no effect in the low-dose. There were similar findings in the female rat liver, with effects (liver weight, preneoplastic foci, and hepatocellular tumors) being observed only at the highest dose, providing further evidence for a threshold for the liver tumors.

Mammary Gland Adenomas

While not observed in the male rats, Kano *et al.* reported a statistically significant increase in mammary gland adenomas in female rats at high doses. Adenomas were not observed in female rats in the drinking water studies conducted by NCI and Kociba *et al.* Mammary gland tumors were not significantly increased in male rats in any of the bioassays. Like subcutis fibroma, mammary gland adenomas are a benign, commonly observed, and spontaneous neoplasm of F344 rats. [38]

[38] Boorman GA et al. Mammary gland. pp. 295–313. In: Boorman GA, Eustis SL, Elwell MR, Montgomery CA, MacKenzie WF (Eds). Pathology of the Fischer Rat. Academic Press, San Diego, CA (1990).

Rat Inhalation Study (Kasai et al., 2009)

For its assessment of inhalation cancer risk, EPA modeled seven tumor types found at an increased incidence in the male rats in the inhalation bioassay by Kasai *et al.* Statistically significant increases in tumors were seen at the highest dose of 1250 ppm for peritoneal mesothelioma, hepatocellular adenomas, and nasal squamous cell carcinomas and at the middle dose for peritoneal mesothelioma and subcutis fibroma. No statistically significant increase was reported for renal cell carcinoma or Zymbal gland adenoma. The potential association of hepatocellular adenomas, subcutis fibroma, and mammary gland adenomas with 1,4-dioxane exposure was discussed in relation to the evidence reported by Kano *et al.* The incidence of Zymbal gland adenomas at the highest dose in the inhalation bioassay by Kasai *et al.* are neither statistically nor biologically significant, since the relevance to humans is uncertain. Likewise the increase in renal cell carcinomas reported by Kasai *et al.* are not statistically significant and are limited to the highest dose.

Nasal Squamous Cell Carcinomas (SCC)

As with the nasal tumor data for the study by Kano *et al.*, EPA has extrapolated the observable tumor incidence data and inappropriately applied a linear extrapolation (see Figure I-5, page 354 of the draft risk evaluation). Kasai *et al.* reported 0/50 nasal SCC in control male rats, 0/50 in the low-dose group (50 ppm), and 1/50 at the mid-dose group (250 ppm). The high-dose group had 6/50 tumors. In an earlier study, Torkelson *et al.* reported a lack of nasal cytotoxicity and nasal tumors in Wistar rats exposed intermittently to 111 ppm 1,4-dioxane for 2 years.[39] As noted earlier, these data suggest that nasal toxicity requires exposure above a threshold effective dose, whether by direct contact alone or a combination of direct contact and internal exposure.

Peritoneal Mesothelioma

The incidence of peritoneal mesothelioma occurs spontaneously in male F344 rats[40] and seldom occurs directly in toxicological studies.[41] Rather it occurs as a result of changes in other organ systems. Accordingly, Kasai *et al.* reported a statistically significant increase in peritoneal mesotheliomas in male, but not female, F344 rats at the two high doses. This sex difference is likely due to the occurrence of tunica vaginalis mesotheliomas (TVM) which grow into the

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39 Torkelson *et al.* 1974.

40 Haseman *et al.* 1984; Hall WC. Peritoneum, retroperitoneum, mesentery, and abdominal cavity. Chapter 6:63-69. In Boorman GA, Eustis SL, Elwell MR, Montgomery CA, MacKenzie WF (Eds). Pathology of the Fischer Rat: Reference and Atlas. San Diego, CA: Academic Press (1990).

41 Zimmerman B. Peritoneum, retroperitoneum, mesentery, and abdominal cavity. Chapter 8:71-77. In Suttie AW (Ed). Boorman's Pathology of the Rat: Reference and Atlas. Second Edition. Academic Press (2015).

peritoneal cavity. TVMs are a commonly occurring tumor in these male rats, but are rare in humans, and likely are not relevant to this risk evaluation.[42]

Evidence for a Threshold MOA for 1,4-Dioxane Carcinogenicity

Despite the evidence supporting a non-mutagenic, threshold MOA for tumor formation resulting from exposure to 1,4-dioxane in laboratory animals, the draft risk evaluation concludes that --

> The relationship between cell proliferation, hyperplasia, and 1,4-dioxane mediated tumor formation has not been established. Though several publications . . . do provide evidence of cytoplasmic vacuolar degeneration and hepatocellular necrosis in rat and non-neoplastic lesions, the animal data does not support a dose-response relationship between cell proliferation, hyperplasia, and liver tumors in rat and mouse studies. Kociba *et al.* (1974) reported hepatic degeneration and regenerative hyperplasia at or below dose levels that produced liver tumors, but incidence for these effects was not reported. Therefore, a dose-response relationship could not be evaluated, and the events cell proliferation and hyperplasia are not supported by available data. Finally, the doses in hepatotoxicity studies where cytotoxicity and cell proliferation were observed were greater than cancer bioassay dose levels. Integrating data across studies, dose-response relationships between cytotoxicity and tumor formation are not well established in the rat and mouse data and are inconsistent among bioassays and across exposure duration.[43]

EPA's conclusion does not accurately capture the scope of information detailing key events, how these key events support a proliferative MOA that is threshold in nature, and the dosimetry around the key events. Other than the female mice liver adenoma response reported in Kano *et al.*, the cancer findings and key events demonstrate adequate dose-response relationships that EPA modeled for establishing $BMCL_{10}$ values from which to derive a threshold-based cancer toxicity value. As noted in the draft risk evaluation and the 2014 and 2017 publications by Dourson *et al.*, issues with the Kano *et al.* mice data preclude the use of this information for risk assessment. The following discussion provides additional perspective on the evidence supporting key events and a proliferative-regenerative repair MOA for the major tumors (liver and nasal mucosa) that are relevant to humans and are the more sensitive endpoints for development of cancer toxicity values.

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42 Haber LT *et al.* Assessment of human relevance and mode of action for tunica vaginalis mesotheliomas resulting from oral exposure to acrylamide. *Regul Toxicol Pharmacol* 53(2):134–149 (2009).

43 Draft Risk Evaluation for 1,4-Dioxane, at 101.

In the drinking water bioassay, Kano *et al.* report that "[a] significant increase in the incidence of hepatocellular foci was observed in the 1000 and 5000 ppm exposed males and the 5000 ppm- exposed females." These exposures result in doses that exceed the limit of metabolic saturation in the rate which has been estimated to be between 30 and 100 mg/kg/day. In the 2008 subchronic (90-day) drinking water study, moreover, the same research group observed respiratory tract and liver lesions (including single cell necrosis and centrilobular swelling) in rats and mice at exposures of 1600 ppm and higher.[44] Further evaluation of the lesions reported in this study by re-reading the slides would help to further characterize these lesions and more firmly establish the dose-response relationship. However, attempts to access the slides from the Japanese bioassay were not successful. [45]

Dourson *et al.* provide important perspective on the Kano *et al.* (2009) study regarding information relevant to dosimetry and MOA --

> While the non-neoplastic lesions found in the NCI (1978) slide reread that we show in this paper were not reported in mice from one long-term study (Kano *et al.* 2009), the same Japanese investigators did report hepatic hyperplasia (later changed to altered hepatocellular foci) in an earlier report of this same 2- year study . . . Moreover, mice in the Kano *et al.* (2009) study showed hepatocellular injury as evidence by an enhanced cytolytic release of liver enzymes . . . at doses of about 140-1400 mg/kg/day [unpublished results].[46]

Unpublished data from Kociba *et al.* described a number of important key events supporting a regenerative repair and/or direct mitogenic response to 1,4-dioxane exposure that is correlated with saturation of the metabolic pathway 4-DX metabolism at 100 mg/kg/day (Figure 7). For example, increased foci occur at a greater frequency and earlier than do liver tumors. This phenomena was also observed for vacuolar degeneration and necrosis, with both occurring with greater frequency and lower doses than the high-dose liver tumor (HCA) response. This illustrates a good dose-concordance for key events explaining a tumor promotion apical outcome.

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[44] Kano H *et al.* Thirteen-week oral toxicity of 1,4-dioxane in rats and mice. J Toxicol Sci 33: 141-153 (2008).

[45] Dourson *et al* 2017.

[46] Dourson *et al.* 2014, at 399.

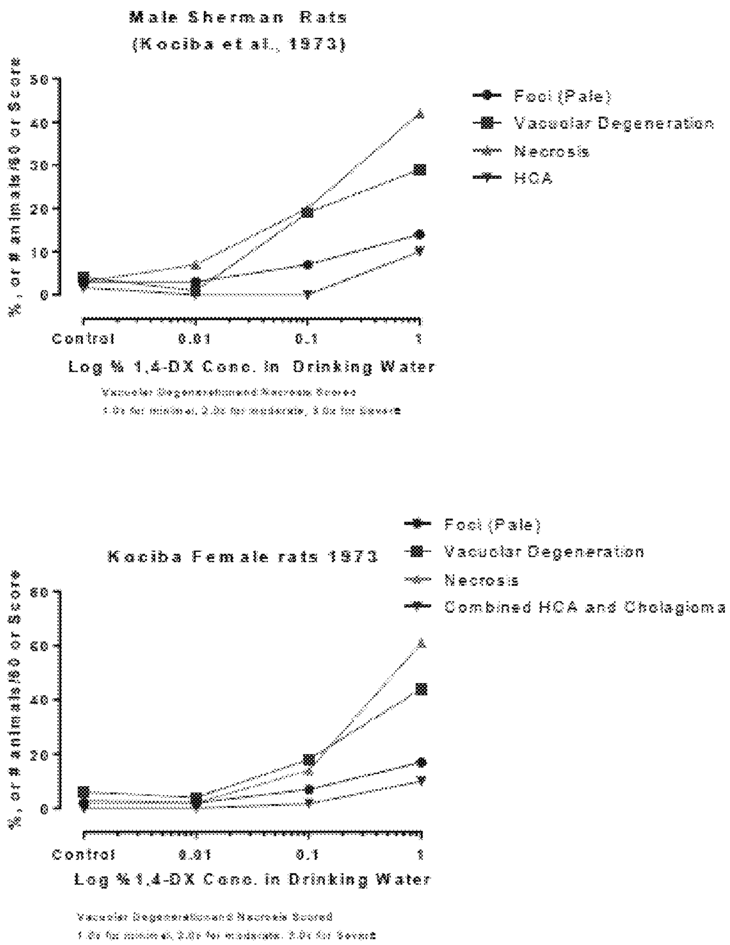

Figure 7. Liver effects from Kociba *et al.* (unpublished)[47]

Kasai *et al.* (2009) also report a number of histopathological observations that illustrate cell proliferation consistent with tumor promotion (Figure 8).

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[47] Estimated doses were 0, 9.6(0.01%), 94 (0.1%) and 1015 (1.0%) mg/kg/day for males and 0, 19, 148 and 1599 mg/kg/day for females. The unpublished results from Kociba *et al.* will be submitted to EPA as part of ACC's supplemental comments.

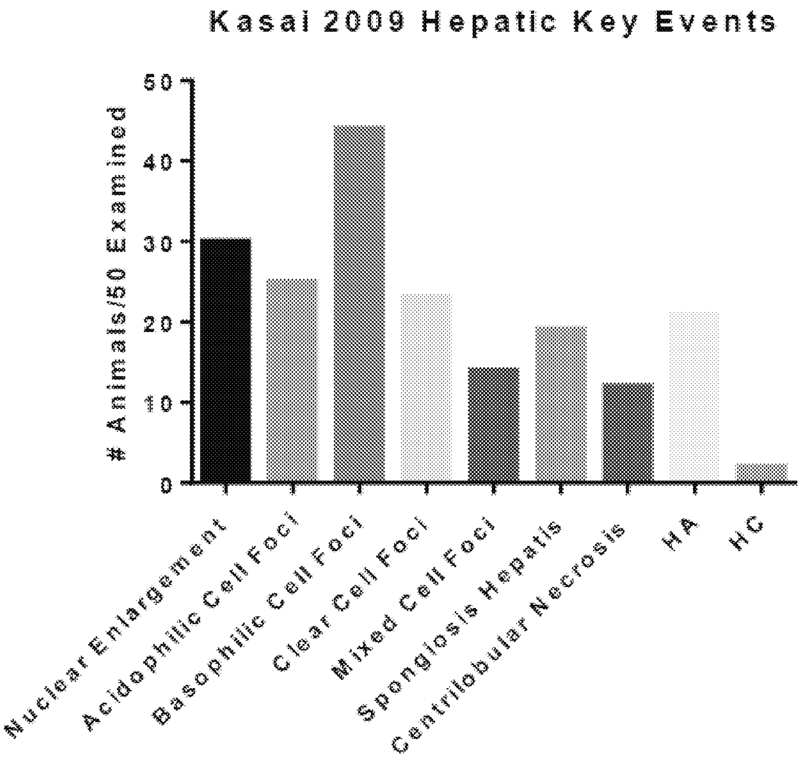

Figure 8. Histological findings in rats exposed to 1250 ppm 1,4-dioxane in Kasai *et al.*[48]
(HA- adenomas, HC – carcinomas)

The various focal lesions shown in Figure 8 represent hyperplasia induced by either direct stimulation of proliferation (which we have shown in a recently completed 90-day mouse drinking water study) or indirectly stimulated by regenerative repair.

A similar pattern of regenerative repair histopathological lesions is seen in the data reported for effects in nasal tissue (Figure 9). These data show a number of the key events that EPA identified that clearly show tissue injury with regenerative repair (metaplasia) occurring in both respiratory and olfactory mucosa.[49]

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48 Kasai et al. 2009, at 893 (Table 3).

49 Table 4-7a (page 102) of the Draft Risk Evaluation for 1,4-Dioxane.

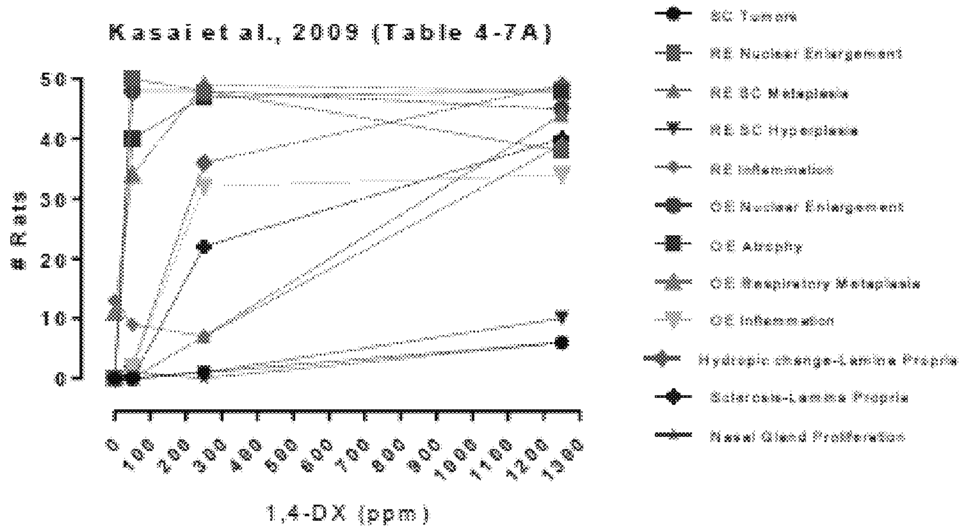

Figure 9. Key events for rat nasal tumors in Kasai *et al.*[50]

While the draft risk evaluation suggests that nuclear enlargement in the respiratory and olfactory epithelium observed after 2-years of lifetime chronic exposure is not adverse,[51] it represents a chronic changes that is just as likely indicative of key event changes observed with tumor promotion resulting from hepatocyte polyploidy.[52] Overall, these key events that precede nasal tumors in terms of dose-response all support a regenerative repair MOA and a threshold modeling of the cancer risk.

Summary of ACC Sponsored Study Results

In reviewing the animal evidence for a regenerative hyperplasia MOA for 1,4-doxane, Dourson *et al.* noted that "dose response and temporal concordance for noncancer precursors to tumors were clearly evident for rats and generally supportive for mice."[53] The authors noted that while extensive liver toxicity could be found in the slides available from the NCI bioassay, Kano *et al.*

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50 Kasai et al 2009, at 893 (Table 3).

51 Draft Risk Evaluation for 1,4-Dioxane, at 89.

52 Jevtic P *et al.* Sizing and shaping the nucleus: mechanisms and significance. *Cur Opin Cell Biol* 0:16-27 (2014); Thoolen B *et al.* Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol* 38:5S-81S (2010).

53 Dourson *et al.* 2017, at 53.

reported more tumors and an absence of liver histopathology in the 2-year bioassay.[54] The lack of liver histopathology was unexpected since the authors had reported clear evidence of liver effects in an earlier 13-week study.[55]

To better define the key events associated with tumor formation in the laboratory animals, ACC sponsored a subchronic drinking water study in female mice.[56] Mice consumed drinking water containing 0, 40, 200, 600, 2000 or 6000 ppm 1,4-dioxane (approximately 0, 10, 40, 120, 360 and 1,000 mg/kg/day, respectively) for 90-days. Interim sacrifices were conducted after 7 and 28 days of exposure. Standard in-life measurements, clinical chemistries, H&E, biomarkers including BrdU, Caspase 3, GSTP+, blood levels of 1,4-dioxane and HEAA, and mRNA were evaluated.

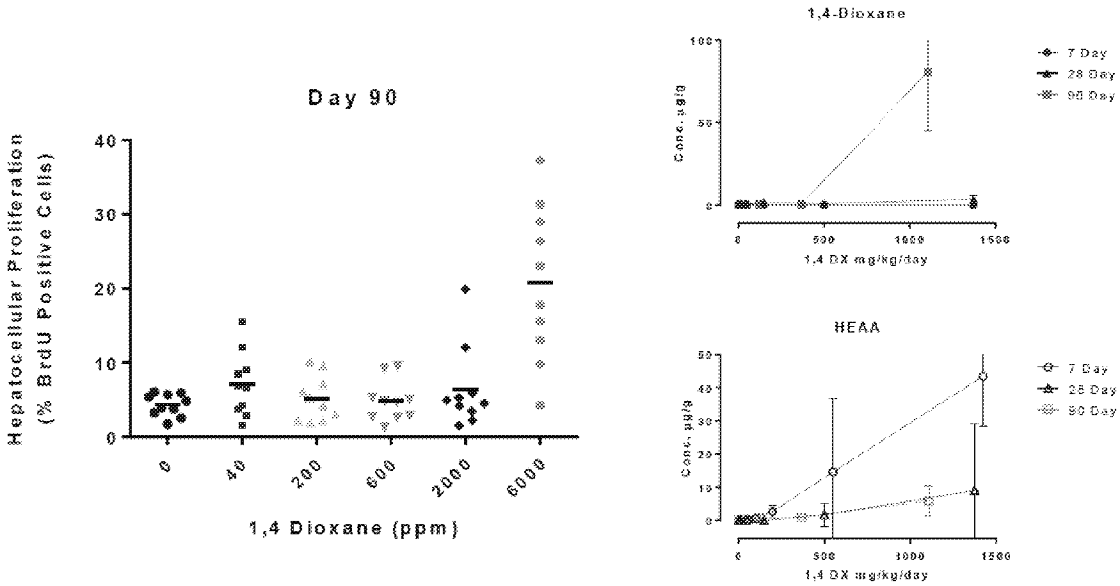

Figure 10. Increase cell proliferation in female mice correlates with an increase in blood 1,4-dioxane concentrations (ACC sponsored study)

There was an increase in liver weights in animals exposed to 2000 and 6000 ppm 1,4-dioxane for 90 days with insignificant changes in liver weights at lower exposures. H&E observations of glycogen-accumulation-related vacuolization, centrilobular hypertrophy, and centrilobular GST-P staining, panlobular increase in proliferation (BrdU), and elevated Caspase 3, were all tightly

54 The difference may be due to a change in histopathological analysis noted by Kano *et al.* whereby hepatic hyperplasia were reclassified as hepatocellular adenomas and altered hepatocellular foci (Kano *et al.* 2009, at 2777).

55 Kano H *et al.* 2008.

56 TERC 2019. The results of this recently completed study will be provided to EPA as part of ACC's supplemental comments.

linked to the accumulation of 1,4-dioxane in the blood, especially at the 90-day time point. There was no evidence of liver injury, either from clinical chemistry endpoints or histopathology unlike what was observed in a previous 2-year bioassay.[57] However, the researchers did not observe an early-phase increase in BrdU incorporation consistent with a direct mitogenic stimulus at exposures that exceeded the demonstrated metabolic saturation levels after 90-days of exposure. No such stimulus was observed at lower exposures at 90 days or at any exposure at the earlier time points. The threshold observed in this study was 2000 ppm. The increase in the BrdU response correlates with an approximate 4.5 fold increase in 1,4-dioxane blood levels after 90-days of exposure at 6000 ppm (Figure 10).

As part of the study, ACC also sponsored a transcriptomics analysis of the livers using the TempO-Seq platform (BioSpyder Technologies). The results of this analysis demonstrate an increase in xenobiotic metabolism, a subtle yet significant dose- and time-responsive increase in mitotic cell cycle and cellular proliferation, and a decrease in complement cascade processes and lipid metabolism. The signals for proliferative response only occur at exposures of 2000 ppm or greater, while alterations related to xenobiotic metabolism occur as low as 600 ppm. Activation of DNA damage response and/or repair mechanisms was not evident at any of the concentrations and time points evaluated. As illustrated in Figure 11, there are no significant changes in signaling pathways/gene sets at the transcriptomic level at concentrations below 600 ppm.

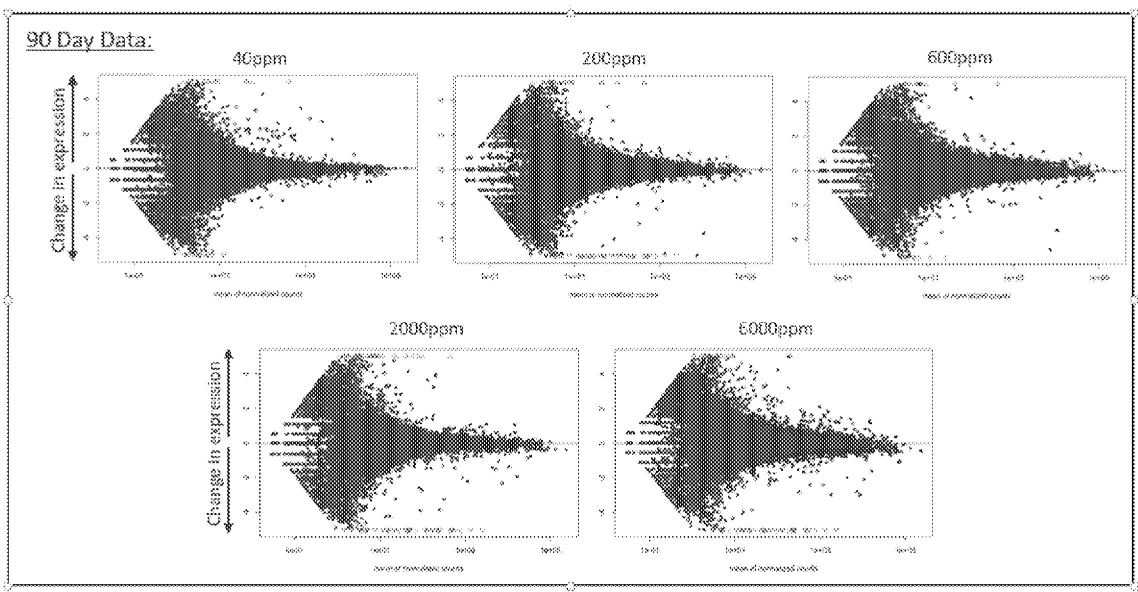

Figure 11. Differential gene expression data after 90 days of exposure in female rats in the ACC sponsored study (red dots designate significant up- or down-regulation of individual genes.)

[57] Dourson *et al.* 2014